Peptide Resonance Assignments

Suggested reading:
Book: “NMR of Proteins and Nucleic Acids”, Kurt Wüthrich

Book: “NMR of Macromolecules: A Practical Approach”, G. C. K. Roberts, Chapter 4


Why are chemical shifts important?

Essential for understanding further studies, e.g. structural data, dynamics, interactions
Provide information about protein structure and function which can be interpreted in advance of determining a three-dimensional structure.
For proteins the patterns of chemical shifts are diagnostic of secondary structure: alpha-helices or beta-strands (CSI: Wishart D. and Sykes B. Biochemistry 31: 1647–1651).
Provide an easy way to monitor the state of the protein under different conditions: ligation state, conformational state (folded/unfolded), solution condition (variable pH, temperature, pressure, etc.).
Ligand binding constants can be deduced from the analysis of chemical shift as a function of the added ligand concentration.
Chemical shift changes help define what parts of molecules interact.
Chemical Shift Distribution

The \(^1\)H chemical shifts of an amino acid are determined by its covalent structure (amino acid type), the neighboring residues (amino acids flanking in the primary sequence), interactions with spatially close amino acids and solvent in the folded protein.

The random coil shifts are those of the amino acid in an unfolded protein.

<table>
<thead>
<tr>
<th>Residue</th>
<th>NH</th>
<th>dH</th>
<th>pH</th>
<th>Others</th>
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<tr>
<td>Gly</td>
<td>8.39</td>
<td>3.97</td>
<td>1.39</td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>8.29</td>
<td>4.35</td>
<td>1.39</td>
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<tr>
<td>Val</td>
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<td>4.18</td>
<td>2.13</td>
<td>(\text{CH}_2) 0.97, 0.94</td>
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<tr>
<td>Ile</td>
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<td>4.23</td>
<td>1.90</td>
<td>(\text{CH}_2) 1.48, 1.19</td>
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<tr>
<td>Leu</td>
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<td>4.38</td>
<td>2.05, 1.65</td>
<td>(\text{CH}_2) 0.95</td>
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<tr>
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<td>1.80</td>
<td>(\text{CH}_2) 0.94, 0.90</td>
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<td>2.28, 2.02</td>
<td>(\text{CH}_2) 2.03, 2.03</td>
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<td>2.10</td>
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<td>2.22</td>
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<td>Glu</td>
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<td>Arg</td>
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<td>4.38</td>
<td>1.90, 1.79</td>
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<td>Asn</td>
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<td>4.75</td>
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<td>(\text{NH}^+) 7.52</td>
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<td>Gln</td>
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<td>4.52</td>
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<tr>
<td>Cys</td>
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<td>4.69</td>
<td>3.26, 2.96</td>
<td>(\text{CH}_2) 2.13</td>
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<tr>
<td>Trp</td>
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<td>4.70</td>
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<td>Phe</td>
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<td>4.66</td>
<td>3.22, 2.99</td>
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<tr>
<td>Tyr</td>
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<td>4.60</td>
<td>3.13, 2.92</td>
<td>(\text{CH}_2) 7.39</td>
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<tr>
<td>His</td>
<td>8.41</td>
<td>4.63</td>
<td>3.26, 3.20</td>
<td>(\text{CH}_2) 7.14</td>
</tr>
</tbody>
</table>

The methyl groups and amide protons can be used to assess the “foldedness” of a protein. In an unfolded protein NH chemical shifts are between 7.5-8.5ppm and for methyl groups are between 0.8-1.2ppm. For a globular folded protein look for dispersion in the methyl groups up-field (below) 0.5ppm and NH groups down-field (above) 8.5ppm.

Methyl groups are shifted from their random coil position by spatially close aromatic groups, interaction known as “ring-current shifts”. If a protein is all alpha-helical, e.g. membrane proteins, or has few (<10%) aromatic residues (Phe, Tyr and Trp) then it is harder to assess the folded state of a protein using the dispersion of the methyls.

Backbone amide protons (NH) are up-field shifted through hydrogen bonding. Generally, in beta-sheets the hydrogen bonds are stronger as compared to alpha-helices and the amide protons are moved further away from the random coil position.

The chemical shifts for side-chain protons vary less from the random coil shifts, especially for solvent exposed long chain amino acids, e.g. Lysine and Arginine, and ring current shifts are the major effect in causing dispersion.
Screening of proteins by 1D $^1$H NMR

In the early days of the Structural Genomics programs, groups from the North East Structural Genomics (NESG) and Joint Center for Structural Genomics (JCSG) consortia showed that the quality of the 1D $^1$H spectrum of a small protein correlated with the ability to obtain a high resolution X-ray crystal structure (Page et al., PNAS (2005) 102:1901–1905; Rossi et al., Biomol NMR (2010) 46:11–22). Wuthrich’s group suggested 4 classes of quality for the 1D $^1$H spectrum:

(a) Good spectrum. Well dispersed with sharp lines.

(b) Good spectrum. Well dispersed but broader lines than in (a).

(c) Poor spectrum. Very broad spectrum but still shows dispersed methyls and NHs.

(d) Bad spectrum. No dispersion in the methyl and NH regions.

The size of the proteins studied ranged from 10kD to 65kD. So while the class (C) spectra are considered ‘bad’ for small proteins, as it would suggest oligomerization, for larger proteins (>30kD) the spectrum would be ‘normal’. All but class (d) proteins were set-up for extensive crystal trials. The results were that proteins exhibiting 1D $^1$H spectra from classes (a) and (b) yielded diffraction of crystals to a high resolution (<3 Å), proteins from classes (c) and (d) resulted in crystals that diffracted to a much lower resolution (>5 Å).
As the protein size increases so does the number of proton resonances. For proteins (peptides) less than 30-50 amino acids, $^1$H multi-dimensional NMR can be used for assigning resonances. For a small protein (100 residues) there will be typically around 1000 $^1$H, $^{13}$C and $^{15}$N resonances to assign. To simplify spectra and analysis, $^{13}$C and $^{15}$N isotope labeling are used.
Homonuclear $^1$H methods: Sequential assignment procedure

Wüthrich and coworkers (early/mid 1980’s)
1. Identification of amino acid side-chain spin-systems.
2. Identification of neighboring residues in the amino acid sequence.
3. Suitable combinations of the results from 1 and 2 which provides individual resonance assignments in the primary structure of the protein.

K. Wüthrich 1983 Biopolymers 22, 131-138

Because of the peptide bond there is no $^1$H-$^1$H J-coupling between different amino acids, so each amino acid is its own individual spin system.

Use $^1$H, $^1$H COSY and TOCSY spectra to identify all the resonances within the individual spin system. The pattern of the chemical shifts compared to the random coil values for individual amino acids are used to identify the amino acid type.

Then using $^1$H, $^1$H NOESY spectra to link the amino acids together identifies the position of the amino acids within the sequence.

Spin System

A spin system is a group of spins that are connected to each other by scalar (through-bond) spin-spin couplings (J).

In proteins each amino acid is composed of one or more spin systems with the peptide bond separating one amino acid from another.

Spin systems of the non-labile H atom in H-Cα-R can be classified as AX (H-CH: Gly), AMX (H-CH2: Ser, Cys, Asp, Asn), etc.
2D COSY (Correlation Spectroscopy), DQF-COSY
(Spin system Identification)

2D $^1$H, $^1$H COSY correlates all $^1$H resonances that are scalar coupled. The spectrum is symmetrical with the intensity of the cross peaks dependent upon the coupling constant.

It can be used to identify which NH$^i$ resonances are bonded to H$\alpha^i$ resonances and so how many backbone NH resonances are present. All the predicted peaks are not necessarily observed due to weak couplings, obscured by solvent, noise, overlap or degenerate peaks.

COSY spectrum of $\kappa$-conotoxin in H2O

The H$^N$-H$\alpha$ region is known as the fingerprint region as it is used to identify all the HN present. The aromatic side-chain protons can be unambiguously identified by running a COSY spectrum in D$_2$O so that the labile HN and NH$_2$ protons are exchanged for deuterium.
2D TOCSY (TOTal Correlation SpectroscopY)
(Spin System Identification)

Unlike the $^1$H,$^1$H COSY spectrum which only gives a correlation to a directly $^3$J coupled proton, in the 2D $^1$H,$^1$H TOCSY experiment the magnetization is transferred during a multi-pulse spin-lock period along the spin system as long as protons are scalar coupled. The TOCSY mixing time can be varied between 15 to 100ms with shorter values giving a COSY-like spectrum and longer values giving complete spin-systems even for Lysine. The transfer of magnetization is not uniform but sinusoidal in the TOCSY mixing period. So, for example, correlations to the amide proton of an Arginine, at short mixing times you may see $^N$H-$\alpha$ and $^N$H-$\beta$ and at long mixing times you may only see $^N$H-$\beta$, $^N$H-$\gamma$, and $^N$H-$\delta$ BUT not the $^{H-N}$-$\alpha$.

For a lot of systems the COSY and TOCSY spin systems will look identical, even for very different amino acids, e.g. Asp/Asn vs. Phe/Tyr/Trp. For the aromatic residues, the backbone and aromatic spin systems are not connected to each other through scalar couplings so they appear as two separate spin-networks.
COSY and TOCSY connectivity patterns

Side-chain assignments involve “matching” the expected patterns and typical chemical shift ranges. Some connectivity patterns are not unique and can only eliminate some possible assignments. Essential to record both COSY and TOCSY to identify the resonance type e.g. Hβ and Hγ can be unambiguously identified by comparing COSY and TOCSY.

In real data, overlapping or missing cross-peaks are common. Connectivity pattern may not exactly match predicted.

Variability in connectivity patterns

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Variability in connectivity patterns

Structure induces chemical shift changes which perturbs the pattern and induces overlap. But the data has to be consistent with the amino-acid spin system or the assignment is probably incorrect.

Look at the TOCSY fingerprint region (NH correlations) to identify the spin states.
Aliphatic $^1$H chemical shifts of all amino acids obtained from a database of 13 proteins. The bars extend one standard deviation in either direction from the mean value.

### Summary of expected HN fingerprint region

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Characteristics in the fingerprint region of TOCSY spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly</td>
<td>Possible doublet arising from non-degenerate $\mathrm{H}\alpha_1$, $\mathrm{H}\alpha_2$</td>
</tr>
<tr>
<td>Ala, Thr</td>
<td>Upfield single cross peak from $\mathrm{H}^\mathrm{N}$-$\mathrm{H}\beta$ for Alal or $\mathrm{H}^\mathrm{N}$-$\mathrm{H}\gamma_2$ for Thr</td>
</tr>
<tr>
<td>Val, Ile, Leu</td>
<td>$\mathrm{H}^\mathrm{N}$-$\mathrm{H}\alpha$-$\mathrm{H}\beta$-$\mathrm{H}\gamma$-$\mathrm{H}\delta$ coupling system is observable with long mixing time. Val two $\gamma$-methyls observed around 0.9 ppm and one $\mathrm{H}\beta$ around 2.13 ppm</td>
</tr>
<tr>
<td>Ser, Asp, Asn, Cys, Trp, Phe, Tyr, His</td>
<td>No $\mathrm{H}\gamma$ proton; $\mathrm{H}\beta$ downfield shift of $\sim$2.5 ppm; resonances arising from aromatic rings will normally need NOESY spectrum for assistance</td>
</tr>
<tr>
<td>Met, Gln, Glu</td>
<td>$\mathrm{H}\beta$ upfield shift of $\sim$2.5 ppm.; $\mathrm{H}^\mathrm{N}$-$\mathrm{H}\gamma$ where $\mathrm{H}\gamma$ is downfield of $\mathrm{H}\beta$</td>
</tr>
<tr>
<td>Lys, Arg</td>
<td>$\mathrm{H}^\mathrm{N}$-$\mathrm{H}\gamma$ where $\mathrm{H}\gamma$ is upfield of $\mathrm{H}\beta$</td>
</tr>
<tr>
<td>Pro</td>
<td>No $\mathrm{H}^\mathrm{N}$ correlation in TOCSY fingerprint</td>
</tr>
</tbody>
</table>
2D NOESY (Nuclear Overhauser Spectroscopy)
(Sequential and through space correlation)

The 2D $^1$H, $^1$H NOESY experiment correlates protons that are close in space (< 5Å) and the intensity of the cross peaks $\propto 1/r^6$. Whilst a NOESY spectrum can contain cross peaks also seen in a TOCSY or COSY spectra the mechanism of magnetization transfer is different, NOESY is via cross relaxation and is a through-space effect, TOCSY/COSY is through J-coupling and is a through-bond effect.

The NOE builds up during the mixing time ($\tau_M$) and for proteins $\tau_M$ is usually 100-150 ms, whilst for peptides it is usually longer, 200-500 ms. The reason for reducing the mixing time for proteins is to prevent spin-diffusion. In a three spin system if A is close to B, B close to C, but A and C are greater than 5 Å then with long mixing times the magnetization can be transferred from A to B and then B to C giving rise to a cross peak between A and C. This is known as spin diffusion and occurs often in proteins because of the increased number of protons present.

In the assignment process a $^1$H$_N$ may correlate with $^1$H$_\alpha$ and $^1$H$_\alpha$-1. So in the cartoon on the right, the $^1$H for residue B gives a NOE to both its $^1$H$_\alpha$ and the previous residue’s $^1$H$_\alpha$.

Notice how many inter-residue HN-aliphatic proton NOEs there are (i.e. how many NOEs that do not coincide with a TOCSY correlation) and how few aliphatic-aliphatic inter-residue NOEs there are.
Link identified amino acids (spin systems) using NOESY data. It is virtually impossible to align the main chain atoms of two adjacent amino acid residues in a protein so that there is not at least one pair of inter-residue distances between main chain hydrogens that are significantly less than the NOE detection limit (~5Å).

**Sequential inter-residue NOEs**

Most short inter-proton (\(^{1}H^N\), \(^{1}H\alpha\), \(^{1}H\beta\)) distances in proteins are between directly adjacent amino acid residues; intense NOEs indicate adjacent amino acids.

Less intense \(i, i+2\) and \(i, i+3\) NOEs (“non-sequential”) also observed and useful, particularly in well-defined secondary structures.

<table>
<thead>
<tr>
<th>Distance (Å)</th>
<th>(j-i=1) (%)</th>
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<tbody>
<tr>
<td>(d_{HN}(i,j)) ≤ 2.4</td>
<td>98</td>
</tr>
<tr>
<td>≤ 3.0</td>
<td>88</td>
</tr>
<tr>
<td>≤ 3.6</td>
<td>72</td>
</tr>
<tr>
<td>(d_{HN}(i,j)) ≤ 2.4</td>
<td>94</td>
</tr>
<tr>
<td>≤ 3.0</td>
<td>88</td>
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<tr>
<td>≤ 3.6</td>
<td>76</td>
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<tr>
<td>(d_{HN}(i,j)) ≤ 2.4</td>
<td>79</td>
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<tr>
<td>≤ 3.0</td>
<td>76</td>
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<tr>
<td>≤ 3.6</td>
<td>66</td>
</tr>
<tr>
<td>(d_{HN}(i,j)) ≤ 3.6, (d_{HN}(i,j)) ≤ 3.0</td>
<td>99</td>
</tr>
<tr>
<td>(d_{HN}(i,j)) ≤ 3.6, (d_{HN}(i,j)) ≤ 3.4</td>
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</tr>
<tr>
<td>(d_{HN}(i,j)) ≤ 3.0, (d_{HN}(i,j)) ≤ 3.0</td>
<td>90</td>
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<table>
<thead>
<tr>
<th>Distance</th>
<th>(\alpha)-helix</th>
<th>3(\beta)-helix</th>
<th>(\beta)</th>
<th>(\beta)</th>
<th>turn I</th>
<th>turn II</th>
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<tr>
<td>(d_{HN})</td>
<td>3.5</td>
<td>3.4</td>
<td>2.2</td>
<td>2.2</td>
<td>3.4</td>
<td>2.2</td>
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<tr>
<td>(d_{HN}(i,j+2))</td>
<td>4.4</td>
<td>3.8</td>
<td>3.6</td>
<td>3.3</td>
<td>3.1-3.4</td>
<td>3.8-3.7</td>
</tr>
<tr>
<td>(d_{HN}(i,j+3))</td>
<td>3.4</td>
<td>3.3</td>
<td>3.4</td>
<td>3.6</td>
<td>2.6</td>
<td>2.4</td>
</tr>
<tr>
<td>(d_{HN}(i,j+4))</td>
<td>4.2</td>
<td>2.8</td>
<td>2.6</td>
<td>4.3</td>
<td>4.2</td>
<td>4.3</td>
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<tr>
<td>(d_{HN}(i,j+2))</td>
<td>2.8</td>
<td>2.6</td>
<td>4.3</td>
<td>4.2</td>
<td>2.6</td>
<td>2.4</td>
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<tr>
<td>(d_{HN})</td>
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<td>2.9-4.4</td>
<td>2.9-4.4</td>
<td>2.9-4.4</td>
<td>3.7-4.7</td>
<td>3.6-4.6</td>
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<tr>
<td>(d_{HN}(i,j+3))</td>
<td>2.5-4.4</td>
<td>3.1-5.1</td>
<td>3.6-4.6</td>
<td>3.6-4.6</td>
<td>3.6-4.6</td>
<td>3.6-4.6</td>
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</table>
The Backbone Walk (NOESY fingerprint region)

Consider a TOCSY spectrum that correspond to **Ala**, **Asn**, **Gly** and **Leu**. We also know that we have **Gly-Leu-Ala** in the peptide, but no other combination:

All spins are seen in TOCSY (long mixing time). The NOESY will have both intra-residue correlations (blue circles), as well as inter-residue correlations (red circles), which allows us to find which residue is next to which in the peptide chain. So in the NOESY for Ala there are two correlations to Hα – its own Hα(i) and to the Leu Hα(i-1). The Leu has correlations to its own Hα(i) and to Gly Hα1(i-1) and Hα2(i-2). This confirms the Gly-Leu-Ala sequence.

LJP33 Peptide Sequence: LDKE\text{X}VYFCHLDIIW
\text{X} = \text{Aminoisobutyric acid}
Assignment of the side-chains protons in aromatic and NH$_2$ groups

Protons of aromatic groups: Tyr, Phe, Trp and His; and side chain amide protons of Gln and Asn residues are not scalar coupled to the rest of the side chain. Identification of which amino acid they arise from is reliant on correlations in the NOESY spectra.

Aromatic and side chain $^1$H chemical shifts of all amino acids obtained from a database of 13 proteins. The bars extend one standard deviation from the mean value.

**Phe/Tyr/His**
As the aromatic side-chain protons of Phe and Tyr are non-labile they are usually identified in peptides dissolved in D$_2$O.

DQFCOSY and TOCSY:
Tyr: Intense cross peak between H$_\delta$ and H$_\epsilon$. No additional peaks are observed.
Phe: The H$_\delta$-H$_\epsilon$ cross peak is more intense than H$_\epsilon$-H$_\zeta$. H$_\delta$-H$_\zeta$ cross peak observed in TOCSY spectrum.

NOESY:
Tyr or Phe: Intense NOE between H$_\delta$ and H$_\beta$
His: Intense NOE between H$_\delta2$ and H$_\beta$
Asp/Gln side chain NH$_2$ protons

COSY:
Strong COSY cross peak in H$_2$O between the germinal amide protons.

NOESY:
Weak NOEs with H$\delta$-H$\beta$ (Asn) and H$\epsilon$-H$\gamma$ (Gln)

Assignment of N24 side chain NH2 in $\kappa$-conotoxin

TOCSY (black) is used to identify the H$^N$-H$\beta$ correlations for N24 and the H$\delta$1-H$\delta$2 correlation. NOESY (blue) is used to get H$\delta$-H$\beta$ NOEs to identify the amino acid.
**Trp**

**COSY/TOCSY:**

- H$_2$O: Cross peak between H$\varepsilon$1-H$\delta$1
- H$_2$O/D$_2$O: Cross peaks between H$\zeta$2/H$\eta$2/H$\zeta$3/H$\varepsilon$2

**NOESY:**

- H$_2$O: Cross peak between H$\varepsilon$1-H$\zeta$2
- H$_2$O/D$_2$O: Weak NOEs between H$\beta$-H$\delta$1 and H$\beta$-H$\varepsilon$3

**Assignment of W3 in MutD peptide**

- **NOESY (red), TOCSY short mixing time (green), TOCSY long mixing time (navy blue)**

**H$\varepsilon$1** – TOCSY correlation identifies the H$\delta$1 proton (this is not coupled to anything else in the ring by NOEs nor J-couplings)

**H$\varepsilon$1** – NOESY correlations to both H$\delta$1 and H$\zeta$2. The assignment of the H$\zeta$2 gives a starting point to assign the ring protons.

**H$\zeta$2** – in the TOCSY short mixing time the strongest correlation will be the next proton along in the ring, i.e. H$\eta$2, the weaker correlation is to next proton, H$\zeta$3. In the TOCSY long mixing time there is one further correlation, which is to the H$\varepsilon$3 proton.

**H$\varepsilon$3** – in the NOESY spectrum (top panel) see correlations to H$\beta$ and H$\alpha$ protons. If there were multiple Tryptophans in the sequence this would be the way to identify which aromatic ring is connected to which amino acid.

**H$\delta$1** – NOESY correlations to H$\beta$ and H$\alpha$. 
Prolyl cis/trans isomerization

The X-Pro peptide bond mostly adopts the trans conformation. This is identified by a strong NOE between the \( H_d1/H_d2 \) (3.2 - 4.0 ppm) and the \( H\alpha \) proton of the preceding residue (X). In the cis conformation the \( H\delta(Pro) - H\alpha(X) \) NOE is weak and a large \( H\alpha(Pro) - H\alpha(X) \) NOE is observed.

Problems in Sequential Assignment Procedure (Wuthrich’s)

With increasing size of peptide/protein spectral degeneracy can preclude complete COSY/TOCSY pattern identification (and so amino acid identification).
Also local environment effects on \( J \)-coupling and relaxation/dynamics lead to variability in COSY patterns.
Main Chain Directed approach (MCD)

Wand/Englander and coworkers (*Biochemistry*, 26(19), 1987 5953-5958)

1) Use J correlated spectra to first identify H^N-H_α-H_β ("NAB") units
   -the H^N region of COSY spectra is usually less crowded
   -individual amino acid type identification not attempted at this point

2) Align the NAB units sequentially using the NOESY spectra
   Pattern recognition routines employed to search for well-established patterns of NOEs in the following order: helix, anti-parallel sheet, parallel sheet, turns and loops.
   Once all the backbone coupling units have been aligned sequentially and categorized by secondary structural element, determination of amino acid type of several side chains permit the defined elements of secondary structure to be aligned with the primary sequence

Advantages:
No initial reliance on identity of amino acid to establish connectivity.
Amenable to automation (pattern matching algorithms)

Cons:
Relies on the presence of regular secondary structures and unlikely to work for proteins with large loops or other less regular secondary structures

Tips for resonance assignment:

Print out the molecular structures of all 20 Amino Acids before starting to assign.

Look for unique or easily identifiable spin systems such as Glycine, Alanine, Threonine, Valine, etc. and use any of these residues as starting points for sequential assignments.

Prolines can be identified by lack of NH spin systems in the TOCSY fingerprint region. Additionally, the X-Pro peptide bond mostly adopts a trans conformation that leads to strong NOEs between the Pro-H_δ and the H_α proton of the preceding residue (X).

Try to use both sequential assignment (Wuthrich) and MCD approach to assign: Try to identify the spin system in TOCSY fingerprint if possible, if not identify the NAB unit and then link them using the NOESY data. During the sequential assignment process look for characteristic NOEs indicative of regular secondary structure.